

Hypothalamic Neuroendocrine Correlates of Cutaneous Burn Injury in the Rat: I. Scanning Electron Microscopy^{1,2,3}

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SCOTT, D. E., G. M. VAUGHAN AND B. A. PRUITT, JR. *Hypothalamic neuroendocrine correlates of cutaneous burn injury in the rat: I. Scanning electron microscopy.* BRAIN RES BULL 17(3) 367-378, 1986. Rats were given a standard scald burn of 60% of the body surface or only a sham burn and were sacrificed at intervals from 6 hr to 14 days later. Serum thyroxine (T₄), free thyroxine index (FTI) and triiodothyronine (T₃) were depressed compared to values in respective shams as early as 6 hr post-burn. T₄ and FTI were less depressed on post-burn days (PBD) 2-3 than on PBD 1 and then exhibited a further fall. T₃ remained depressed through PBD 14. Pineal melatonin content was elevated at 6 hr and returned to the normal daytime range in subsequent samples. The ventral portion of the diencephalon was prepared for scanning electron microscopy. Only in the burned rats and beginning on PBD 2, large numbers of supraependymal neurons (SEN) appeared in the ventricular space attached to the inferior walls and floor of the third cerebral ventricle. Transmission electron microscopy was used to confirm the neuronal nature of the SEN. Viewed by scanning electron microscopy, these persisted through PBD 14. SEN were interconnected by cables of their neurites exhibiting varicosities on individual neurites as they passed over perikarya of other SEN. Some SEN were seen to be only partially emerged from the underlying tissue and others were seen to send a thick process into the hypothalamic tissue. These observations indicate that after peripheral injury there is marked plasticity of the brain in an area thought to control the endocrine systems that show abnormalities after such a peripheral injury. The timing, location and nature of these anatomic changes indicate the possibility that at least some aspects of central nervous orchestration of the endocrine metabolic response to injury may be related to the emergence of a neuronal system receiving or sending messages through the cerebrospinal fluid and/or through new neurite circuits along the surface of the third ventricular wall. These structures may appear in response to initial primary hormonal changes and/or may play a role in maintaining the post-injury hormonal milieu manifested in part by a subsequent second fall in serum T₄.

Supraependymal neurons Third cerebral ventricle Varicosities Thyroxine Burn injury Melatonin

THE neuroanatomical organization of the mammalian hypothalamus and its relationship to endocrine control have been thoroughly studied over the last quarter of a century. However, little is known concerning the response of the intrinsic cerebral ventricular system, anatomically associated with the endocrine hypothalamus, to a profound and protracted systemic stimulus. Cutaneous burn injury results in major and long lasting abnormalities of many hormonal systems and other functions controlled by the hypothalamus

[2-5, 16-18, 31, 44, 45, 47-49, 50, 57-59]. The present investigation was designed to assess the ultrastructural responses of the rodent third cerebral ventricular walls and floor after peripheral scald injury, and to observe their temporal relationship to changes in serum thyroid hormones and pineal melatonin as functional neuroendocrine markers.

METHOD

In experiment 1, adult male Sprague-Dawley rats, adapted

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²The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

³In conducting the research described in this report, the investigators adhered to the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and in adherence with the *Guide for the Care and Use of Laboratory Animals*, NIH publication 80-23.

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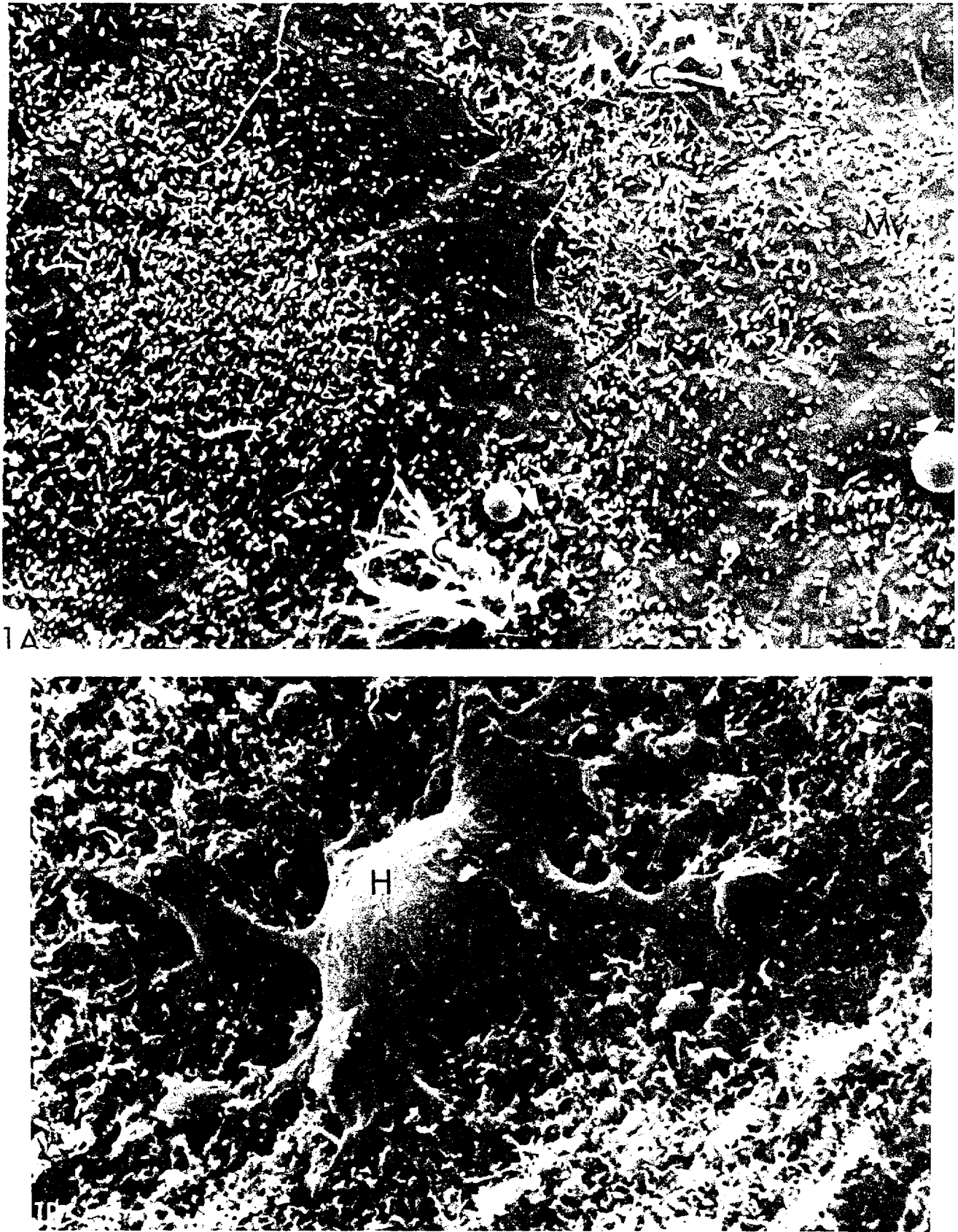


Fig. 1. A: $\times 2200$ scanning electron microgram (SEM) of the ventricular (apical) surfaces of tanyocytes (T) that constitute the floor of the third cerebral ventricle of a normal control rat. The apical surfaces of these specialized ependymal cells feature numerous microvilli (MV), and eccentric tufts of cilia (C), and bulb-like protrusions (arrow). B: $\times 2500$ low magnification SEM of a histiocyte (H) upon the ventricular surface of the lateral recess of a sham rat. This line of phagocytic motile cells is regarded as the resident macrophage of the cerebral ventricular system and becomes quite numerous and active in cases of acute inflammation or infection. These cells are notably different from neurons in that they exhibit flattened palmate processes (P) which are regarded as a structure responsible for their motile properties.

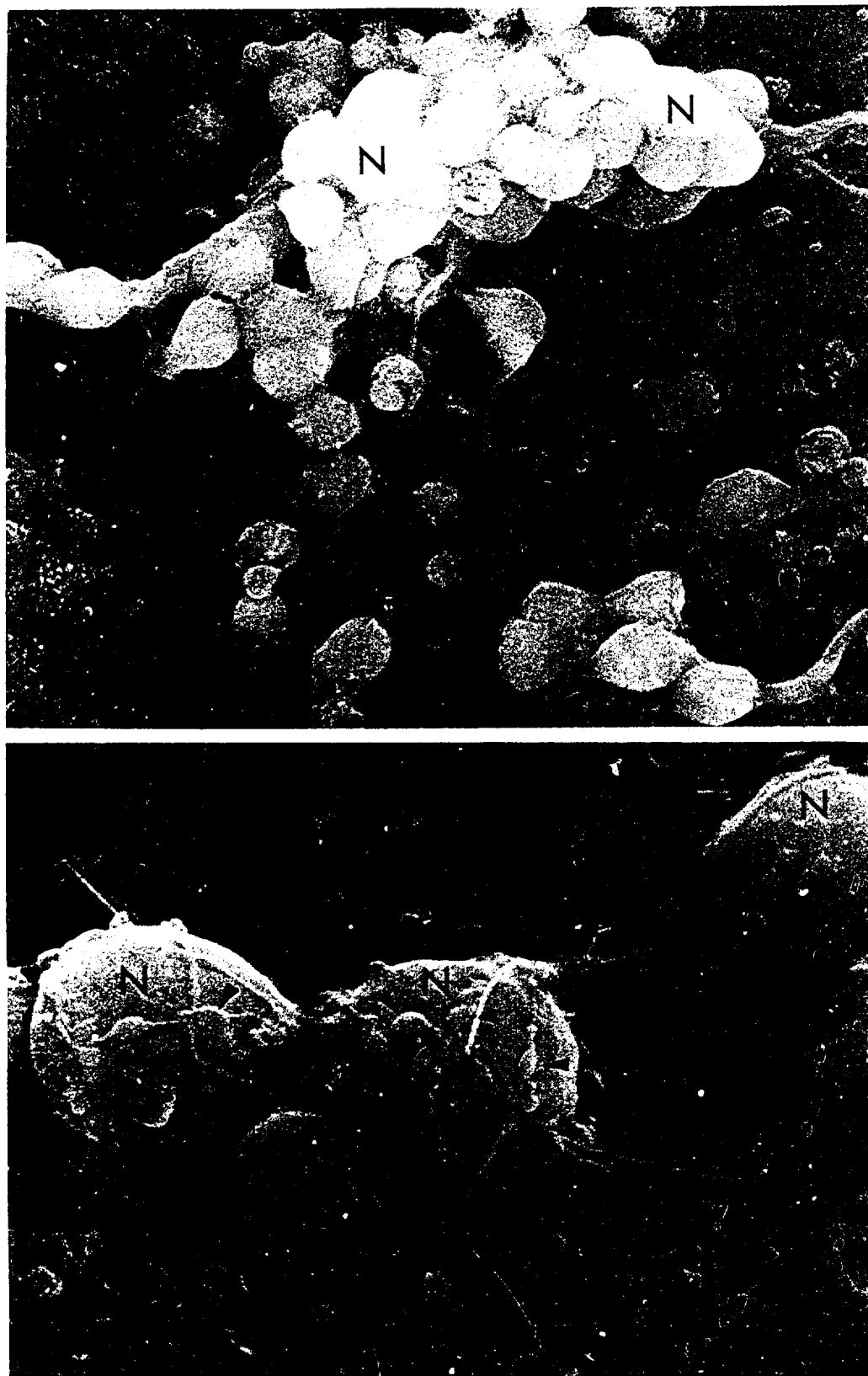


FIG. 2. A: $\times 1200$ low magnification SEM of a large cluster of neurons (N) observed to reside upon the ventricular surface of a burned rat killed 48 hours following acute 60% third degree burn. B: $\times 3500$ mid-range SEM of four neurons (N). Notable here are the presence of numerous processes (arrows) that demonstrate distinct enlargements (varicosities) along their linear axis. Previous investigations have demonstrated these fibers as en-passant synapses.



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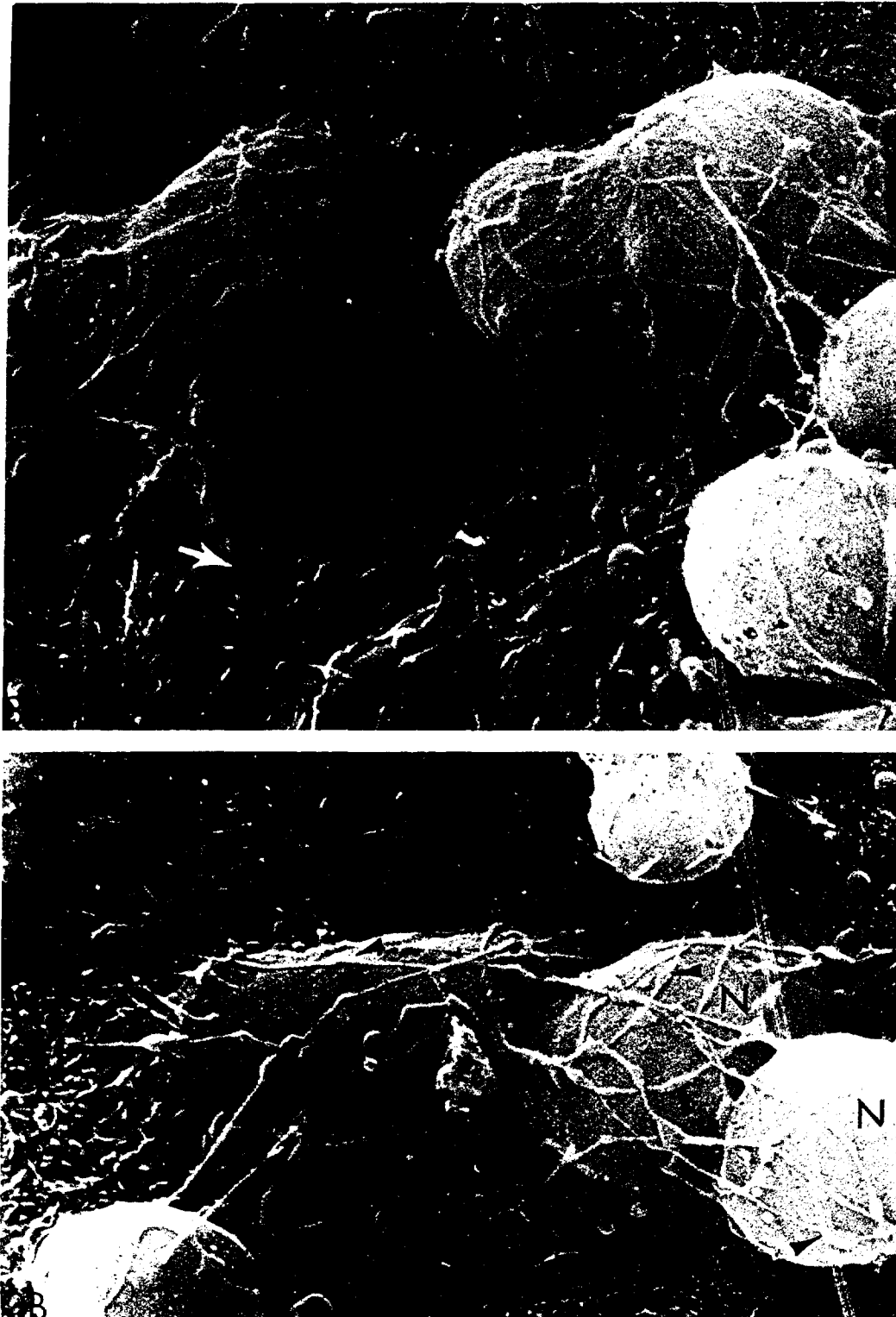


FIG. 3. A: $\times 5800$ SEM of neurons (N) observed in various phases of emergence from the underlying host ventricular wall. In contrast to phagocytic cells of the cerebral ventricular lumen (Fig. 1B), neurons are firmly anchored to the underlying substrate by large, thick penetrating processes (arrow). B: $\times 4900$ SEM of several neurons (N) that exhibit numerous beaded varicosities (arrows) that appear to interconnect neighboring cells. These cells also exhibit variability in size and shape.

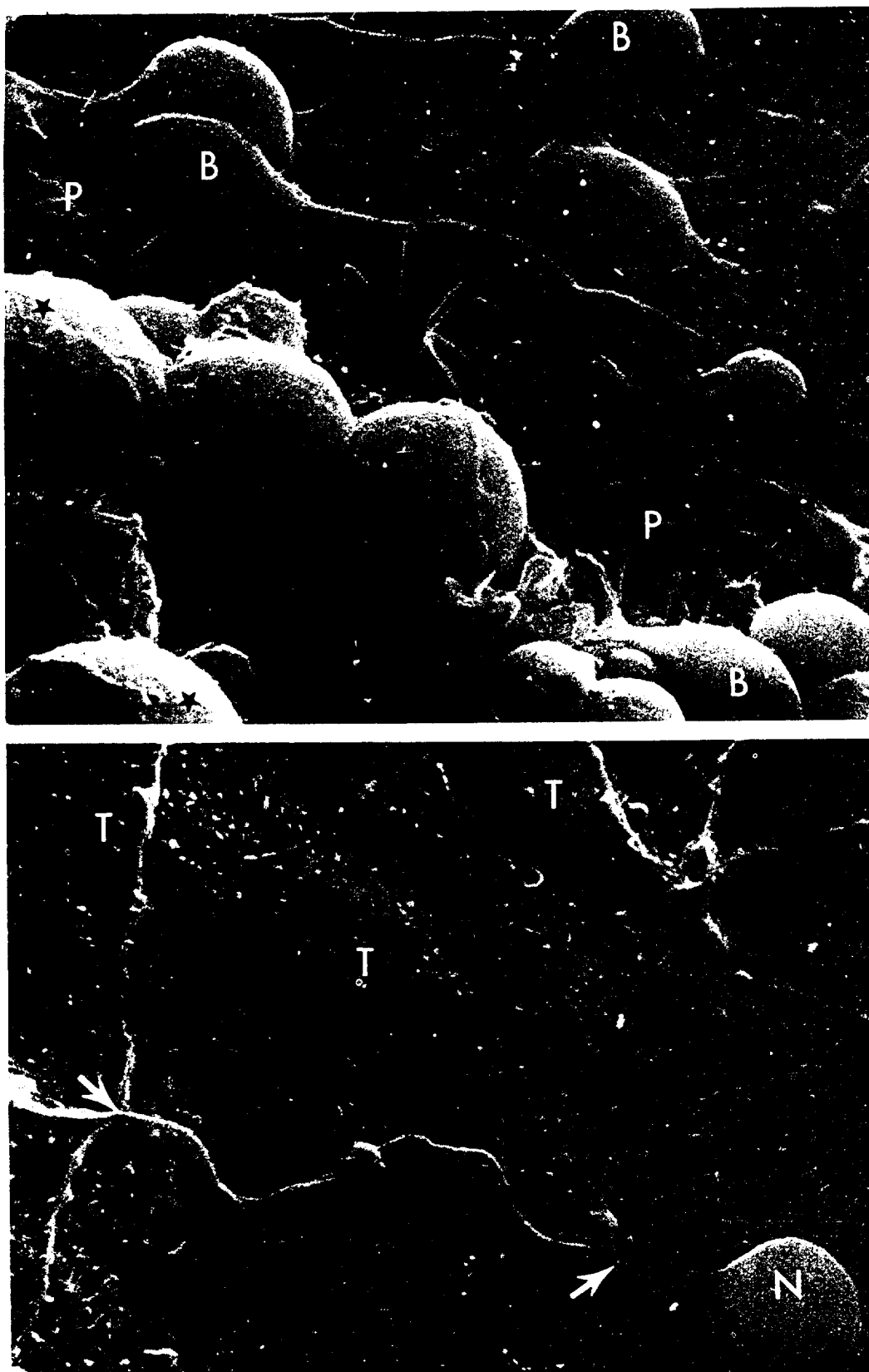


FIG. 4. A: $\times 4000$ SEM of the floor of the third cerebral ventricle of an experimental rat killed 7 days following 60% third degree burn. Both spheroidal (\star) as well as bipolar (B) neurons are observed to reside upon a thick underlying matrix of processes (P). This picture is remarkably different from that observed in normal rats. B: $\times 3000$ SEM of cerebral ventricular floor of experimental rat killed 7 days following acute burn injury. A distinct process from a bipolar neuron (N) is observed to intersect (arrow) with two others that course horizontally over the apical surfaces of tanyocytes (T) that constitute the floor of the third cerebral ventricle.



FIG. 5. $\times 4000$ SEM of floor of third cerebral ventricle of experimental rat killed 48 hours following 60% third degree burn injury. Multiple cell processes are observed to form large cable networks (arrows) that course upon the surface of the third cerebral ventricle and serve to interconnect groups of neurons.

to a light/dark cycle of 14/10 hr (lights on 0600 hr), were fully anesthetized and then subjected to a full thickness scald burn [54] covering a total of 60% of body surface area on the dorsal and ventral sides of the trunk at 0800 hr. Sham rats were anesthetized, the hair on the dorsal and ventral side of their trunks was shaved, and they were exposed through a standard template to room-temperature water. Burned and sham rats were given 30 ml physiologic NaCl IP before exposure of the ventral surface. Groups of 5 burned and 5 sham-burned rats were then decapitated by guillotine at 6, 24 and 48 hours, as well as at 7 and 14 days following the acute burn injury, all at 0800 hr except for the 6-hr time group which was sacrificed at 1400 hr. Nine controls were initially only

anesthetized and were sacrificed at times coincident with the other groups. Trunk blood was taken for serum thyroxine (T_4) and triiodothyronine (T_3) radioimmunoassay (RIA) and *in vitro* radiometric T_3 uptake (T_3 U) with kits from Diagnostic Products, Los Angeles, CA. The product of the T_4 and T_3 U values is the free thyroxine index (FT_4I). Though the FT_4I was slightly more depressed in burned rats than was free T_4 concentration measured by dialysis, mean depressed FT_4I did reliably indicate depressed free T_4 [44], and the FT_4I is thus used as an index of free T_4 . Pineals were frozen for later melatonin analysis [50] with the Rollag antibody [35]. Brains were fixed by immersion in 4% Karnovsky's aldehyde fixative, and the basal medial hypothalamus was blocked and

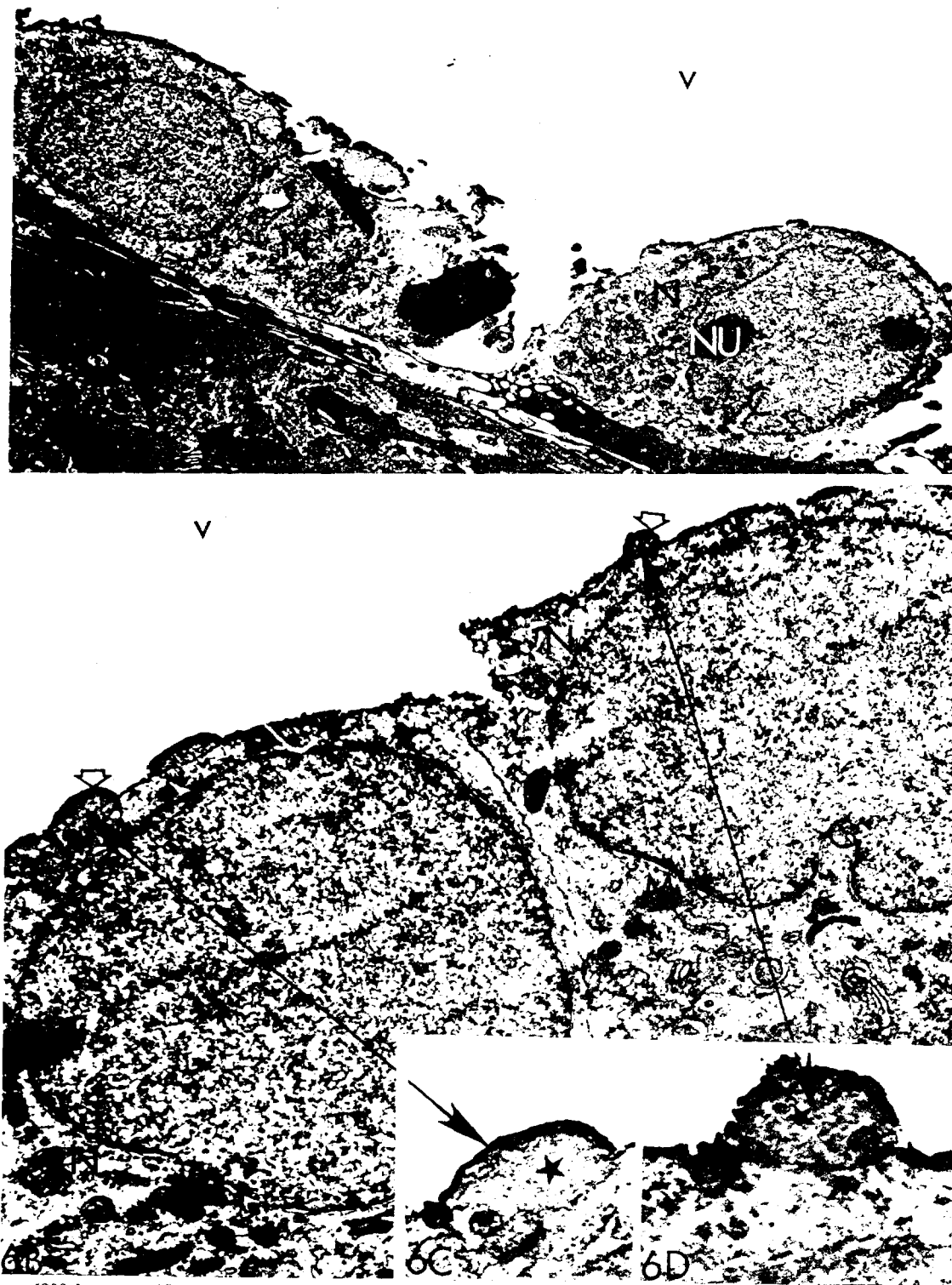


FIG. 6. A: $\times 6800$ low magnification transmission electron microgram (TEM) of previously scanned supraependymal neurons (N) that demonstrate a distinct gold coat from sputter coating due to prior SEM analysis. This unique cell type exhibits distinct nucleoli (Nu), clefting of the nuclear membrane (C) and a rich assortment of cytoplasmic organelles and inclusions which are more clearly observed in section B. V, ventricle. B: $\times 14,200$ TEM showing two supraependymal neurons (N) in contact with the cerebral ventricular lumen (V). Like those in section A, these cells exhibit profiles (arrows) that appear to be pre-synaptic processes that terminate upon their apical plasmalemmata. C, nuclear clefting; G, Golgi cisternae; M, mitochondria. C: (Insert). $\times 28,400$ high magnification TEM of axosomatic neuritic profile (\star) observed in section B which appears to harbor lucent microvesicles. D: (Insert). $\times 28,400$ TEM of axosomatic profile (\star) with rounded lucent vesicles. These profiles correlate well with the picture observed with scanning electron microscopy as seen in Figs. 3A and B.

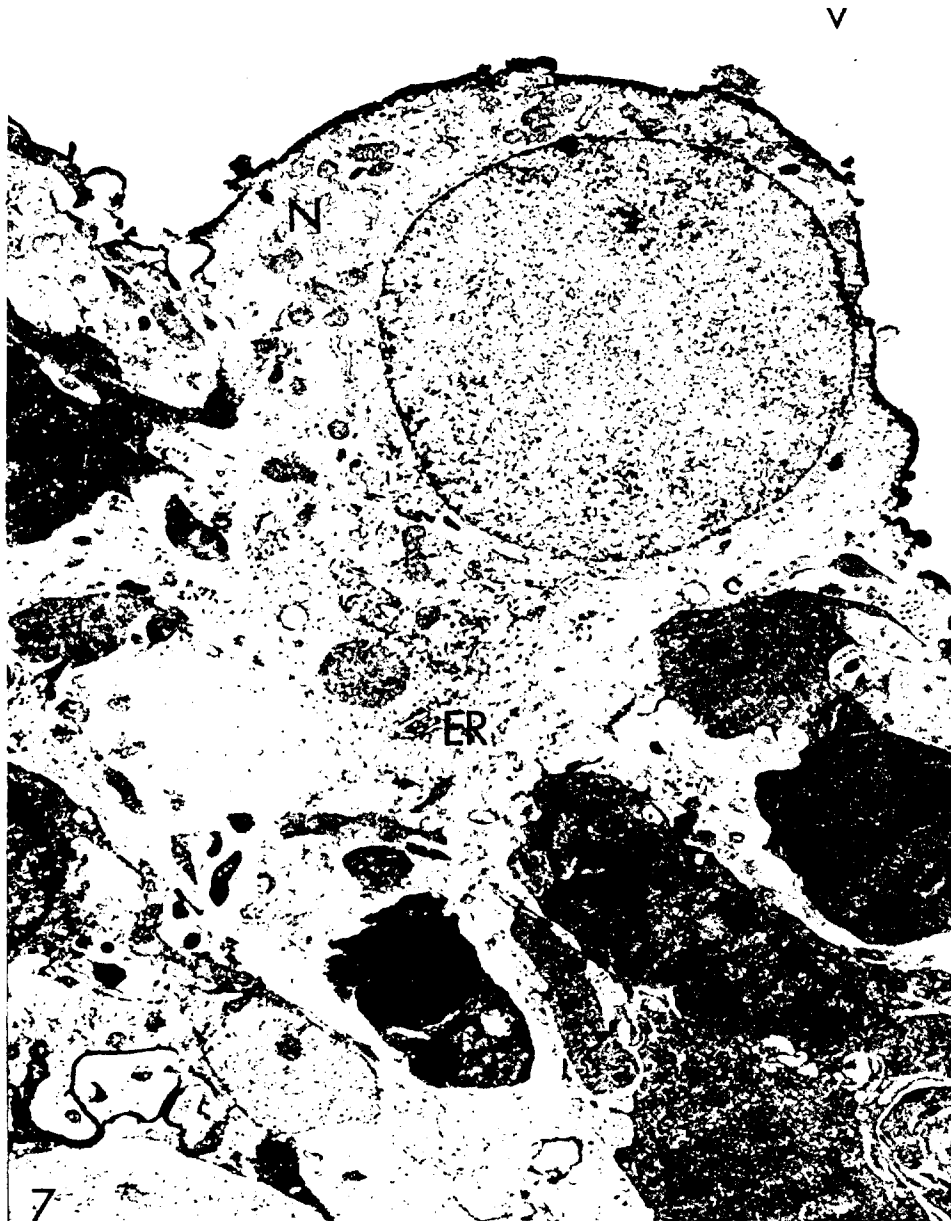


FIG. 7. $\times 12,800$ mid-range TEM of previously scanned supraependymal neuron (N). This cell appears to have been in the process of emerging from the subependymal parenchyma between adjacent ependymal cells (E). Compare with Figs. 2 and 3. ER, rough endoplasmic reticulum; V, cerebral ventricular lumen.

prepared for correlative scanning and transmission electron microscopy. Coded specimens for blind analysis were examined with a Nanolab 2100 scanning electron microscope and at an emission potential of 15 kV. Hormonal data were analyzed by the *t*-test with Bonferroni correction for overestimation of significance due to multiplicity of comparisons [15].

In experiment 2, groups of adult male rats received a 60% burn or sham procedure and were sacrificed 1, 3, or 7 days post-burn in groups of 6–7 animals. All procedures were the same as in experiment 1, except that the pineals and brains were not saved for analysis.

RESULTS

The third cerebral ventricle of the normal rat can be subdivided into distinct regions. The dorsal thalamic wall is characterized by the presence of cuboidal ependymal cells exhibiting a wealth of cilia that are known to be responsible for the local dynamics and flow of cerebrospinal fluid. At the junction between the thalamic and hypothalamic walls, this nap of cilia upon the ventricular borders of cuboidal ependymal cells diminishes in density. These ependymal cells give way to a specialized population of cells called tanycytes that constitute the lining of the lower walls (lateral recesses)

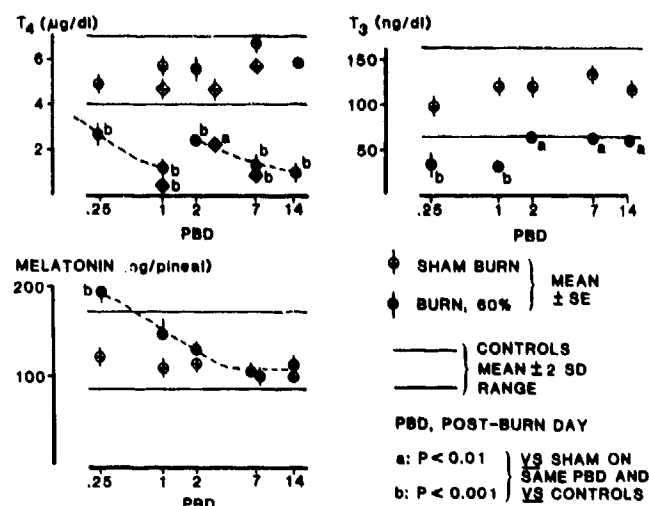


FIG. 8. Serum concentrations of thyroxine (T_4) and triiodothyronine (T_3) and pineal content of melatonin after cutaneous burn injury. PBD 0.25 represents the time point 6 hr after injury. The dashed lines indicate single-exponential regressions of data from burned animals in experiment 1. The diamond-shaped symbols (T_4) are from experiment 2, and all other data are from experiment 1. In experiment 2, the T_4 mean on PBD 3 was greater ($p < 0.01$) than that on PBD 1. After combining experiment 2 burn group T_4 data with T_4 data of experiment 1 at the same or nearest respective PBD, the combined PBD 2+3 group T_4 mean was higher than that for the combined group at PBD 1 ($p < 0.001$) and at PBD 7 ($p < 0.01$). The log scale for time is used only graphically for the convenience of saving space and allowing the early data to be seen easily.

and floor of the third cerebral ventricle (the dorsum of the median eminence). These cells are essentially devoid of cilia (Fig. 1A) and, instead, exhibit a thick feltwork of microvilli. The surfaces of tanycytes are arranged into a hexagonal pattern with microvilli which are most apparent along the interface between apposed cells. The ventricular surfaces in normal rats are relatively devoid of other cell types with the exception of occasional histiocytes individually distributed throughout the ventricular wall surface (Fig. 1B).

At 6 hours post-burn, few visible changes were evident upon the ventricular floor and walls of experimental animals. The ultrastructural appearance of the cerebral ventricular wall 24 hours following burn was still undisturbed. However, by 48 hours a remarkable neuroanatomical alteration was consistently detected in all of the experimental animals killed at this time period. Large numbers of supraependymal neuron-like cells (SEN) could be observed upon the floor of the third cerebral ventricle and in the lateral recess as well (Figs. 2, 3, and 4). This species of cell was distinct in that it exhibited delicate bouton-like processes which correlated well with neurites described in other investigations. These neurites with distinct varicosities appeared to terminate upon neighboring cells (Figs. 3B and 4B). A large proportion of these processes exhibited multiple enlargements (varicosities) along their linear axes, reminiscent of axonal processes. Thick cables of cell processes interpreted to be neurites were observed to course over the surface of the ventricle and served physically to interconnect clusters of SEN (Fig. 5).

The SEN that had emerged upon the ventricular surfaces of experimental rats were distinctly different in their anatomical appearance and organization from those cells that have been identified in previous studies as histiocytes.

TABLE 1

SERUM FREE THYROXINE INDEX (FT₄I)* IN EXPERIMENT 1
[MEAN \pm SE (n)]

	Post-burn day				
	0.25	1	2	7	14
Sham	2.34 ± 0.15 (5)	2.60 ± 0.19 (5)	2.60 ± 0.20 (5)	3.03 ± 0.12 (5)	2.61 ± 0.07 (5)
Burn	1.33† ± 0.27 (5)	0.60‡ ± 0.16 (5)	1.13‡ ± 0.10 (5)	0.64‡ ± 0.17 (5)	0.44‡ ± 0.08 (5)

*FT₄I controls (mean \pm 2 SD range): 1.80–3.23.

† $p < 0.01$, ‡ $p < 0.001$; vs. controls and vs. respective shams.

TABLE 2

SERUM FREE THYROXINE INDEX (FT₄I) AND TOTAL TRIIODOTHYRONINE (T_3) IN EXPERIMENT 2 [MEAN \pm SE (n)]

		Post-burn day		
		1	3	7
FT ₄ I	Sham	1.95† ± 0.22 (7)	1.90* ± 0.17 (7)	2.42† ± 0.16 (7)
	Burn	0.14 ± 0.02 (7)	1.00‡ ± 0.13 (7)	0.52 ± 0.09 (4)
T_3 (ng/dl)	Sham	54.2* ± 7.6 (7)	51.6 ± 5.0 (7)	58.7* ± 5.6 (7)
	Burn	12.4 ± 0.7 (7)	34.3‡ ± 3.4 (7)	29.3 ± 6.6 (4)

* $p < 0.01$, † $p < 0.001$; vs. burn.

‡ $p < 0.05$ vs. burn PBD 1 or 7.

§ $p < 0.05$ vs. burn PBD 1.

SEN were not observed in shams or control rats, whereas histiocytic cells were. SEN appeared to be in different phases of emergence from the underlying periventricular parenchyma (Fig. 4A). Apparently once having emerged, large populations of SEN remained relatively stable and constant and were noted in 7- and 14-day-post-burn experimental rats as well. In the vicinity of emergent SEN, large numbers of neurites were seen to obscure partially the underlying surface of tanycytes and presented a picture far different from that observed in controls or sham rats (Figs. 4A and 5).

Previously scanned samples of the cerebral ventricular walls and floor of experimental rats terminated 48 hours post-burn were embedded with epon and thick 1 micron sections and ultrathin sections were prepared and examined with transmission electron microscopy. TEM analysis confirmed that the population of emergent supraependymal cells observed with SEM were bona fide neurons. The cells exhibited clefting of the nuclear membranes and harbored a

range of cytoplasmic organelles and inclusions to include rough endoplasmic reticulum, Golgi cisternae, polysomes and numerous mitochondria (Figs. 6A,C and 7). A critical criterion for their positive identification as neurons was the presence of axosomatic (pre-synaptic) profiles that were observed to terminate upon their somata. Their axonal profile contained numerous clear microvesicles typical of axosomatic synapses.

Figure 8 shows that serum T_4 in experiments 1 and 2 was significantly reduced at each post-burn time in burned rats compared to respective shams (and in experiment 1 to the controls considered as one group). Consideration of the control values as baseline allows the estimation of an initial 6-hr half-life of T_4 from the control level. Serum T_3 in experiment 1 was also significantly reduced at each time point in burns vs. shams and controls. In experiment 1, FT_4I (Table 1) followed the same pattern as did T_4 (Fig. 8). In experiment 2 (Table 2), FT_4I and T_3 followed the same pattern as in experiment 1, but the difference between PBD 1 and 3 was, in this case, significant for FT_4I and T_3 . Pineal melatonin content in experiment 1 was elevated at 6 hr in burns compared to shams and controls and was down into the normal range by 24 hr. For each hormonal variable in experiment 1, at no time was a sham group mean significantly different from the control group mean.

DISCUSSION

The cerebral ventricular system of the mammalian brain has been the subject of intense analysis for the last decade. A wealth of literature has served to describe the cerebral ventricular surfaces of the rat [13, 14, 20, 22, 28, 30, 38, 56], the hamster [6], the guinea pig [26], the cat [9], the rabbit [51], subhuman primates [10-12, 24, 25, 38-41], and the human as well [38, 41, 43]. The presence of supraependymal neuron-like cells in apparent contact with the lumen of the third cerebral ventricular system and, hence, in the living state necessarily bathed in cerebrospinal fluid is not a finding unique to this investigation. The so-called "liquor-contact" neuron was first described by Vigh-Teichmann and others over 15 years ago [51-53] and occasionally is encountered throughout the cerebral ventricular lumen of normal animals. However, it should be noted that remarkably large accumulations of SEN such as those appearing in this investigation 48 hours post-burn are observed only rarely under normal conditions. An increase in the number of SEN has been noted following bilateral adrenalectomy [27], gonadectomy [29], and the intraventricular infusion of P-chloroamphetamine [36] and after the stereotaxic placement of normal hypothalamic fetal neurografts into the third cerebral ventricular lumen of Brattleboro rats with chronic diabetes insipidus [37,42].

Burn injury is associated with elevated sympathetic activity for weeks after injury, manifested by greatly augmented urinary excretion and plasma concentrations of catecholamines [3, 4, 57, 58]. Elevated serum cortisol without suppressed and even sometimes with elevated circulating corticotrophin values [5, 16, 47] as well as severely reduced serum testosterone with little or no compensatory elevation of luteotrophin (LH) by RIA [5,17], and severe depression of serum LH by bioassay [31] in burned male humans suggests centrally mediated changes in hormonal regulation, i.e., overactivity of the adrenal axis and underactivity of the reproductive axis. Impaired spermatogenesis [17], depressed plasma folliculotrophin, and elevated prolactin concentra-

tions [5] in burned humans also support a central mechanism. Reduced circulating T_4 and T_3 with no compensatory augmentation of or with actual suppression of the serum thyrotrophin (TSH) response to injection of its releasing hormone (TRH) in burn patients is seen in the presence of an elevated metabolic rate [3,4], just the opposite of the metabolic response to true hypothyroidism. Other observations suggesting altered hypothalamic function in such patients include the resetting of temperature control around a higher level, the suppression of serum growth hormone responses to standard provocative stimuli [59], elevation of plasma arginine vasopressin (AVP) disproportionate to plasma tonicity [45], and a reduced nocturnal surge of plasma melatonin [50].

The observed neuroanatomical changes in the ventricular wall and floor overlying the median eminence of the hypothalamus may represent a morphologic basis for the mechanism producing some of the post-injury endocrine changes usually thought to be mediated by the hypothalamus. Precisely how these emergent neuronal structures are related to the endocrine response to burn injury is not yet known. However, it is now clear that the neuronal changes following burn injury are profound enough to include neuroanatomical reorganization in an area that controls abnormally functioning endocrine systems. The major population of periventricular neurons (so-called A12 and A14 groups) that constitute the ventricular wall and floor of this area of the endocrine hypothalamus may be dopaminergic in nature [46]. Our unpublished observations agree with this, in that these cell groups stain exclusively with antisera against the enzyme probe tyrosine hydroxylase, but not dopamine beta hydroxylase or phenylethylamine-N-methyltransferase.

The emergence of a large population of neurons into the cerebral ventricular lumen following peripheral burn injury may represent a dynamic mechanism of neuroanatomical remodeling and plasticity in response to an acute alteration in the endocrine status of experimental animals. For a number of years, the cerebral ventricular system and its product, the cerebrospinal fluid, have been regarded as a trophic mediator and a potential mechanism for the distribution of biologically active molecules throughout the central nervous system [34]. Both serotonergic and dopaminergic neurites have been documented to terminate within the cerebral ventricular lumen and are thought to arise from nerve cell bodies in the raphe and locus coeruleus [1, 7, 8, 20-23, 32, 33]. Although their precise physiological role is not yet understood, it is clear that they represent a potential substratum for the delivery of bioactive molecules into the CSF. The presence of a wide range of physiologically active substances has been well documented in mammalian cerebrospinal fluid [34]. An extensive cutaneous burn injury appears to stimulate a neuroanatomical reorganization and mechanical-plastic change in groups of dopaminergic neurons of the A12 and A14 groups adjacent to the cerebral ventricular lumen. Their emergence upon the floor and walls of the third ventricle may be a means of mobilizing neurons and their secretory products to provide a new pathway of delivery of biogenic amines through the cerebrospinal fluid to adjacent circumventricular organs [19,55]. The role of such neurons may be to modulate the metabolism and activity of adjacent peptidergic systems controlling the anterior pituitary, or vasopressinergic neurons of the supraoptic and paraventricular nucleus. For example, if the mobilized neurons we observed are dopaminergic as are those in the nearest groups that presumably give them origin, they might

provide an extra supply of this neurotransmitter through the CSF to stimulate vasopressin neurons, normally receiving some dopamine input through neurites from other areas [1,46]. This would be consistent with excessive vasopressin secretion observed in burn injury [45].

The morphologic changes we noted were found only on the second post-burn day or later, whereas, the endocrine changes were already present 6 hr post-injury. This suggests that these structural changes may be a result of endocrine changes, an interpretation compatible with the occurrence of similar structural alterations after endocrine manipulation [27, 29, 37, 42]. However, we cannot exclude the possibility that we observed the severe end-stage extent of a morphologic change that began earlier after burn but was not apparent. In such a case, morphologic changes might still be part of the overall central nervous system integrative function that interprets the incoming signal of the presence of a peripheral injury and orchestrates the long-term endocrine and metabolic responses.

Reports of hormonal responses to burn injury and other nonthyroidal illness have often focused on thyroid hormones but with little attention to the early pattern (see [4, 16, 44, 48, 49]). In this study, we have now measured T_4 and T_3 within the first 24 hr after injury and find that they are markedly depressed by 6 hr post-injury. This initial fall of T_4 , T_3 , and FT₄I might be explained partially by a fall in serum proteins (bound hormones) and free hormones consequent to initial extravasation of serum components out of the vascular compartment with dilution of serum from the resuscitation fluid and any subsequently imbibed water. What is interesting is that the FT₄I did not subsequently return toward normal after PBD 2, indicating that after this time there was sufficient suppression of the thyroid axis to prevent the raising of the free T_4 levels toward normal, a situation compatible with the previously reported depressed FT₄I and free T_4 in burned rats on PBD 8 and 14 [44]. In fact, in the present study, a second phase of T_4 and FT₄I fall-off was recorded after post-burn days 2-3. Thus, the emergence of the supraependymal

neurons might play a role in initiating or maintaining suppression of the thyroid axis manifested by the further depression of T_4 and prevention of the return of T_3 levels to the normal range.

Little information is available on the pineal response to burn injury. We have previously reported that beyond 7 days after a 60% burn in rats, neither day nor night values of pineal melatonin were altered compared to values in shams despite the known control of pineal melatonin synthesis by its sympathetic innervation from a pathway originating in the hypothalamus [50]. We now observe that at 6 hr after such a burn, pineal melatonin is elevated significantly above the low level normally seen throughout the light phase, though not to the very high levels (about 1000-2000 pg/pineal) ordinarily obtained in normal rats at night. It is not yet known whether the observed hypothalamic structural changes might be related to subsequent regression of these daytime post-burn pineal melatonin levels in spite of the known persistence of the elevated general sympathetic activity.

Depression of serum T_4 and T_3 and elevated daytime pineal melatonin occurred 6 hr after burn injury in rats. The melatonin change resolved within 1 or 2 days following injury while the depressed T_3 persisted to at least day 14. By day 2, a plethora of supraependymal neurons appeared upon the hypothalamic ventricular surface, preceding a second fall of T_4 and persisting through day 14 after cutaneous burn injury. Future studies of the cytochemistry of these neurons and of their association with individual components of the multifaceted endocrine and metabolic response to burns is expected to shed light on the mechanisms underlying plasticity of the brain and its control of the response of other systems to severe injury.

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